Prevention of Excitotoxicity in Primary Retinal Ganglion Cells by (+)-Pentazocine, a Sigma Receptor-1–Specific Ligand

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Purpose. Sigma receptors (σRs) are nonopioid, nonphencyclidine binding sites with robust neuroprotective properties. Previously, the authors induced death in the RGC-5 cell line using very high concentrations (1 mM) of the excitatory amino acids glutamate (Glu) and homocysteine (Hcy) and demonstrated that the σR1 ligand (+)-pentazocine ((+)-PTZ) could protect against cell death. The purpose of the present study was to establish a physiologically relevant paradigm for testing the neuroprotective effect of (+)-PTZ in retinal ganglion cells (RGCs).

Methods. Primary ganglion cells (GCs) were isolated by immunopanning from retinas of 1-day-old mice, maintained in culture for 3 days, and exposed to 10, 20, 25, or 50 μM Glu or 10, 25, 50, or 100 μM Hcy for 6 or 18 hours in the presence or absence of (+)-PTZ (0.5, 1, 3 μM). Cell viability was measured using the viability and apoptosis detection fluorescein in situ assays. Expression of σR1 was assessed by immunocytochemistry, RT-PCR, and Western blotting. Morphologic appearance of live ganglion cells and their processes was examined over time (0, 3, 6, 18 hours) by differential interference contrast (DIC) microscopy after exposure to excitotoxins in the presence or absence of (+)-PTZ.

Results. Primary GCs showed robust σR1 expression. The cells were exquisitely sensitive to Glu or Hcy toxicity (6-hour treatment with 25 or 50 μM Glu or 50 or 100 μM Hcy induced marked cell death). Primary GCs pretreated for 1 hour with (+)-PTZ followed by 18-hour cotreatment with 25 μM Glu and (+)-PTZ showed a marked decrease in cell death: 25 μM Glu alone, 50%; 25 μM Glu/0.5 μM (+)-PTZ, 38%; 25 μM Glu/1 μM (+)-PTZ, 20%; 25 μM Glu/3 μM (+)-PTZ, 18%. Similar results were obtained with Hcy. σR1 mRNA and protein levels did not change in the presence of the excitotoxins. DIC examination of cells exposed to excitotoxins revealed substantial disruption of neuronal processes; cotreatment with (+)-PTZ revealed marked preservation of these processes. The stereoselective effect of (+)-PTZ for σR1 was established in experiments in which (−)-PTZ, the levo-isomer form of pentazocine, had no neuroprotective effect on excitotoxin-induced ganglion cell death.

Conclusions. Primary GCs express σR1; their marked sensitivity to Glu and Hcy toxicity mimics the sensitivity observed in vivo, making them a highly relevant model for testing neuroprotection. Pretreatment of cells with 1 to 3 μM (+)-PTZ, but not (−)-PTZ, affords significant protection against Glu- and Hcy-induced cell death. σR1 ligands may be useful therapeutic agents in retinal diseases in which ganglion cells die. (*Invest Ophthalmol Vis Sci.* 2007;48:4785–4794) DOI:10.1167/iovs.07-0343

Sigma receptors (σRs) represent unique nonopiate, nonphencyclidine binding sites in mammalian brain and peripheral organs, distinct from other known receptors.[1] To date, two types of sigma receptors, distinguishable by biochemical and pharmacologic means, have been identified.[2] The cDNA encoding type 1 sigma receptor (*σR1*), the better characterized of the two subtypes, was cloned initially from guinea pig[3] and subsequently from human, mouse, and rat.[4–7] The *σR1* cDNA predicts a protein of 223 amino acids (M_r 25–28 kDa).[3] Ligands for σR1 demonstrate robust neuroprotective properties, particularly against excitotoxic insults, including decreased neuronal responsivity to N-methyl-D-aspartate receptor stimulation,[8,9] attenuation of postsynaptic glutamate (Glu)-evoked calcium influx,[10,11] inhibition of ischemia-induced Glu release,[12,13] and reduced NO production.[9,14]

σR1 expression has been demonstrated in ocular tissues including lacrimal gland,[15] iris–ciliary body,[16,17] lens,[17,18] and retina.[17,19] Using molecular and biochemical methods, we have studied σR1 in mouse retina.[17,20,21] RT-PCR analysis amplified *σR1* in neural retina and the RPE–choroid complex. In situ hybridization studies revealed abundant expression of *σR1* in the ganglion cell layer, inner nuclear layer, inner segments of photoreceptor cells, and RPE cells. Immunohistochemical analysis confirmed these observations. Recent studies using primary cultures of mouse Müller cells localized σR1 to the endoplasmic reticulum and nuclear membranes.[21] These cells and other retinal cell types demonstrate robust σR1 binding activity with an apparent K_d value of approximately 25 nM.[21]

Additional studies from our laboratory have focused on retinal ganglion cells, the second-order neurons of the visual system. Ganglion cells die in several retinal diseases, including glaucoma and diabetic retinopathy.[22,23] Our earlier work showed that σR1 continues to be expressed in neural retina under hyperglycemic conditions and during diabetic retinopathy,[20] making it a promising target for neuroprotection against cell death in these diseases. To test the neuroprotective properties of σR1 ligands in ganglion cells, we first used the rat ganglion cell line RGC-5 to determine whether (+)-pentazocine ((+)-PTZ), a highly selective benzomorphan-based σR ligand,[24] can block RGC-5 cell death induced by excitotoxins such as Glu.[25] The results were promising. Concentrations of Glu required to induce death in this cell line, however, were extremely high (millimolar range), despite the fact that in vivo ganglion cells are sensitive to micromolar (greater than 15 μM) concentrations of Glu.[26] In addition, RGC-5 cells, like many

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transformed cell lines, replicate in culture, which is not a characteristic feature of neurons in vivo. Finally, the RGC-5 cell line does not form extensive processes characteristic of neurons, making it impossible to analyze effects of excitotoxins on neuronal processes. For these reasons, it was essential to determine whether our earlier promising results of neuroprotective effects of (+)-PTZ observed in the RGC-5 cell line could be replicated in a physiologically relevant system. Recently, we established the culture of primary ganglion cells from the mouse retina. Using the immunopanning procedures initially described for rats, we optimized the isolation and purification of these cells from neonatal mouse retina.[27] The cells are positive for neuronal markers and for Thy1, which is considered a specific marker of retinal ganglion cells. In the present study, we found that the primary ganglion cells, like ganglion cells in the intact retina, form extensive neuronal processes and are exquisitely sensitive to glutamate; hence, the model is highly relevant to studies of neuroprotection.

In addition to assessing the efficacy of σR1 ligands in preventing Glu-induced ganglion cell death, we wanted to explore more fully the toxic effects of homocysteine (Hcy) on primary ganglion cells and to determine whether deleterious effects of exposure to Hcy were reversible using (+)-PTZ. Hcy, a nonprotein sulfur amino acid, is a metabolite of the essential amino acid methionine. Modest plasma elevation of Hcy is a risk factor in cardiovascular and neurodegenerative diseases.[28–30] Less is known about the effects of Hcy on retinal function, though several clinical studies implicate Hcy in maculopathy, retinal vein occlusion, open-angle glaucoma, pseudoexfoliation glaucoma, and diabetic retinopathy.[31–39] A recent report of a child with severe hyperhomocysteinemia caused by methionine synthase deficiency demonstrated decreased rod response and RGC loss as analyzed by ERG and visual evoked potential.[40] We have attempted to understand the consequences of elevated levels of Hcy on retinal function using in vitro and in vivo models. In a study using mice, we injected micromolar concentrations of Hcy intravitreally and observed apoptotic RGC death,[26] thus providing the first report of Hcy-induced retinal ganglion cell death in vivo. The present study explored the sensitivity of primary ganglion cells to micromolar concentrations of Hcy and analyzed the role of σR1 in preventing this excitotoxin-induced cell death. The data showed that primary ganglion cells are exquisitely sensitive to the toxic effects of Glu and Hcy; (+)-PTZ can prevent that cell death.

Materials and Methods

Reagents

Reagents used were as follows: medium and reagent (Neurobasal/B27 medium; TRIzol reagent; Gibco-Life Technologies, Rockville, MD); trypsin inhibitor (Roche Applied Science, Indianapolis, IN); papain (Worthington Biochemical, Lakewood, NJ); brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ); goat anti-rabbit IgG and goat anti-rat IgG (both H+L; Affinipure; Jackson ImmunoResearch Laboratories, West Grove, PA); goat anti-mouse IgG-horseradish peroxidase (HRP), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA); enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL); RNA PCR kit (GeneAmp; Applied Biosystems, Branchburg, NJ); *Taq* PCR kit (TaKaRa; Takara Bio Inc., Otsu, Shiga, Japan); sense and antisense primers for σR1 (Integrated DNA Technologies, Coralville, IA); viability assay (Live/Dead Assay; Molecular Probes, Eugene, OR); apoptosis detection kit (ApopTag Fluorescein In Situ Apoptosis Detection Kit; Chemicon International, Temecula, CA); D,L-homocysteine thiolactone hydrochloride (MP Biomedical, Inc., Solon, OH); L-glutamate, (+)-pentazocine, (−)-pentazocine, monoclonal antibodies to β-actin and neurofilament 160 (NF-160), and all other chemicals (Sigma-Aldrich Chemical, St. Louis, MO).

Isolation and Culture of Primary Ganglion Cells

Primary ganglion cells (GCs) were isolated from the retinas of 1- to 2-day-old C57BL/6J mice that were the offspring of breeding pairs purchased from Harlan Sprague–Dawley Inc. (Indianapolis, IN). Care and use of the mice adhered to the principles set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Immunopanning procedures and verification of purity of the cells have been described in detail.[27] Cells were cultured on poly-D lysine and laminin-coated coverslips in medium (Neurobasal; Gibco-Life Technologies) containing 5 μg/mL insulin, 1 mM sodium pyruvate, 0.1 mg/mL transferrin, 60 ng/mL progesterone, 16 μg/mL putrescine, 40 ng/mL sodium selenite, 40 ng/mL triiodothyronine, 1 mM L-glutamine, 60 μg/mL N-acetyl cysteine, 2% B27, 50 ng/mL BDNF, 10 ng/mL CNTF, 10 ng/mL forskolin, 10 ng/mL bFGF, and 0.1 mg/mL BSA.

Immunocytochemical Analysis of σR1

To establish the presence of σR1 in primary GCs, cells were cultured on coverslips, fixed in methanol, and incubated for 3 hours at room temperature with antibody against σR1[25] or antibody against NF 160, a known neuronal cell marker. Proteins were detected using fluorescent dye (Alexa Fluor-488; Invitrogen, Carlsbad, CA)-conjugated anti-rabbit IgG for σR1 and fluorescent dye (Alexa Fluor-488; Invitrogen)-conjugated anti-mouse IgG for NF 160. Samples were washed with PBS, and slides were covered with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenylindole (DAPI) to stain nuclei. σR1 and NF-160 were detected by epifluorescence using a microscope (Axioplan 2; Carl Zeiss, Thornwood, NY) equipped with viewer software (AxioVision; Carl Zeiss) and an HRM camera.

Sensitivity of Primary GCs to Excitotoxic Amino Acids and Treatment with (+)-Pentazocine

To determine the concentration of the excitotoxin that induced 50% cell death, primary GCs, cultured on coverslips in 24-well plates, were exposed to varying concentrations of Glu (10, 20, 25, or 50 μM) and subjected to viability assay (Live/Dead Assay) that uses calcein-acetoxymethyl ester to detect living cells and ethidium homodimer-1 to detect dead cells, according to the manufacturer's instructions. Cells were viewed by epifluorescence using different filters to detect cells with green fluorescence (live) or red fluorescence (dead). Cell viability was confirmed using the apoptosis detection (ApopTag; Chemicon) kit according to the directions of the manufacturer. The kit is based on the detection of DNA strand breaks through terminal dUTP nick-end labeling (TUNEL) analysis. In experiments testing the effects of Hcy (10, 25, 50, 100 μM) on GC viability, TUNEL analysis was used.

The effects of exposure of excitotoxin-treated cells to (+)-PTZ were assessed by treating cells with 25 μM Glu or 50 μM Hcy in the presence of varying concentrations of (+)-PTZ (0.5, 1, or 3 μM). Cell viability was assessed using the apoptosis detection (ApopTag; Chemicon) kit. Treatment paradigms included coculture of cells with excitotoxin and (+)-PTZ for 6 hours or pretreatment of cells for 1 hour with (+)-PTZ, followed by coculture with the excitotoxin and (+)-PTZ for 6 or 18 hours. Cells were examined by epifluorescence using standard fluorescein excitation and emission filters, and data were captured for image analysis. Each field was examined systematically for the presence of green fluorescence, indicative of apoptosis, and data were expressed per total number of cells in the field. In these experiments, at least three coverslips were prepared. Per coverslip, images were captured from at least four fields with the use of a software system (AxioVision; Carl Zeiss). Data were analyzed by one-way analysis of variance using a statistical package (SPSS version 15.0; SAS Institute, Cary, NC). Least significant difference (LSD) was the post hoc test. $P < 0.05$ was considered significant. Additional experiments were

performed in which primary GCs were pretreated for 1 hour with (−)-PTZ (the levo-isomer of pentazocine) or (+)-PTZ (the dextro-isomer of pentazocine) and were then coincubated with 25 μM Glu for 18 hours. In further experiments, live ganglion cells were subjected to differential interference contrast (DIC) microscopy using an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan). Cell bodies and fibers radiating from them were photographed using a camera at 0, 3, 6, and 18 hours (CoolSnap; Photometrics, Tucson, AZ) after treatment with Glu or Hcy in the presence or absence of (+)-PTZ.

Semiquantitative and Real-Time RT-PCR Analysis of σR1 mRNA

To determine whether *σR1* gene expression was altered in primary GCs treated with excitotoxins in the presence or absence of (+)-PTZ, total RNA was prepared from the cells using reagent (TRIzol; Gibco-Life Technologies). RT-PCR was carried out using primer pairs specific for mouse *σR1*.[41] Sense primer (5′-TAT CGC AGT GCT GAT CCA-3′) and antisense primer (5′-TAC TCC ACC ATC CAC GTG TT-3′) corresponded to nucleotide positions 75 to 92 and 520 to 539, respectively, in the cloned mouse *σR1* cDNA (GenBank accession no. AF030198). The expected PCR product size was 465 bp; 18S RNA was the internal standard. RT-PCR was performed in 35 cycles, with a denaturing phase of 1 minute at 94°C, annealing phase of 1 minute at 60°C, and an extension of 2 minutes at 75°C. Twenty microliters of the PCR products were gel electrophoresed and stained with ethidium bromide. Signals were quantified using a phosphorimaging system (Storm; Amersham Biosciences, Uppsala, Sweden), as described.[25]

For real-time RT-PCR, total RNA was extracted using reagent (TRIzol; Gibco-Life Technologies) and was quantified. RNA (5 μg) was reverse transcribed using the RNA PCR Kit (GeneAmp; Applied Biosystems). cDNAs were amplified for 45 cycles (iCycler; Bio-Rad, Hercules, CA) using microliter reaction (iQ SYBR Green Supermix; Bio-Rad Laboratories) and sequence-specific primers for mouse *σR1* (sense primer, 5′-CTC GCT GTC TGA GTA CGT G-3′; antisense primer, 5′-AAG AAA GTG TCG GCT AGT GCA A-3′). The internal reference was hypoxanthine phosphoribosyltransferase 1 (HPRT1), for which the primers were sense primer (5′-GCG TCG TGA TTA GCG ATG ATG AAC-3′) and antisense primer (5′-CCT CCC ATC TCC TTC ATG ACA TCT-3′). Expression of *σR1* relative to HPRT1 was calculated by comparison of C_t values (δ-delta C_t).

Immunoblot Analysis of σR1

Western blotting of σR1 protein in primary retinal ganglion cells followed our published method.[21] After protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes, the membranes were incubated with anti-σR1 antibody (1:500) followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody (1:3000). Proteins were visualized using the ECL Western blot detection system. Membranes were reprobed with mouse monoclonal anti-β-actin antibody (1:5000) as a loading control. The films were analyzed using a digital imaging system (AlphaImager 2200; Genetic Technologies, Miami, FL) as described.[20]

Results

σR1 has been detected in intact retinal tissue including cells of the GC layer.[17] To determine whether the primary GCs used in this study expressed σR1, we performed immunocytochemistry and immunoblotting. The primary GCs developed extensive processes and variably sized cell bodies (Fig. 1A), which are characteristic of GCs in vivo. They are positive for the neuronal cell marker NF-160 (Fig. 1B) and the ganglion cell-specific marker Thy-1.[27] σR1 is detected in primary GCs by immunocytochemistry (Fig. 1C) and by immunoblotting (Fig. 1D). Thus, the cells are a useful and relevant model for studies of the protective role of σR1 ligands.

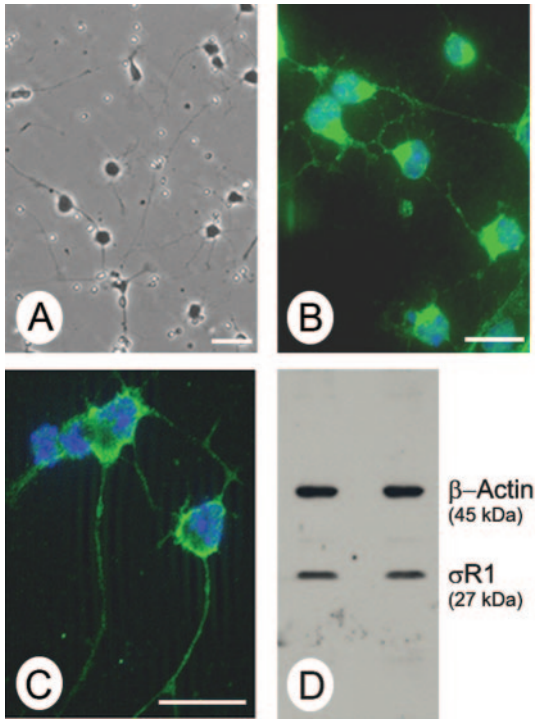

Figure 1. Detection of σR1 in primary GCs isolated from mouse retina. (**A**) Phase-contrast images of mouse primary GCs after 3 days in culture. The cells have long processes extending from the variably sized cell bodies that are characteristic of GCs. (**B**) Immunolabeling of primary GCs with an antibody against NF-160, a neuronal marker detected with fluorescent dye (*green*); nuclei stained with DAPI (*blue*). (**C**) Immunolabeling of primary GCs with an affinity-purified polyclonal antibody against σR1, detected with fluorescent dye (*green*); nuclei stained with DAPI (*blue*). (**D**) Two separate preparations (*left* and *right lanes)* of primary GCs used for immunoblotting with an affinity-purified antibody against σR1 (M_r approximately 27 kDa) and an antibody against β-actin (M_r approximately 45 kDa, internal loading control). Magnification bar, 15 μm.

In vivo, the intracellular concentration of Glu in neurons can be as high as 10 mM; however, the extracellular concentration of Glu must be maintained in the micromolar range to avoid toxicity.[42] To determine whether the primary GCs used in this study exhibited similar sensitivity to extracellular Glu as they did in vivo, the cells were cultured in supplemented medium (Neurobasal; Gibco-Life Technologies) for 3 days and then 6 hours in medium containing increasing concentrations of Glu. Cell viability was assessed using a viability assay (Live/Dead Assay; Molecular Probes; Fig. 2A); living cells were green and dead/dying cells were red. Cells incubated in medium with a final concentration of 10 μM Glu had excellent viability, but in the presence of 25 μM or 50 μM Glu, cell viability decreased by approximately 50% and approximately 80%, respectively (Fig. 2B). Subsequent experiments used 25 μM Glu to induce cell death in the primary GCs. With the use of the viability assay (Live/Dead Assay; Molecular Probes), the number of living cells was determined in primary GCs exposed to 25 μM Glu alone or in primary GCs pretreated for 1 hour with varying concentrations of (+)-PTZ and then coincubated for 16 hours with (+)-PTZ and 25 μM Glu. Pretreatment of cells with 0.5, 1, or 3 μM (+)-PTZ resulted in markedly fewer dead cells than in cells exposed to Glu in the absence of (+)-PTZ (Fig. 2C).

To confirm and extend the findings described in Figure 2, a second cell viability measure, the TUNEL assay, which detects DNA strand breaks in cells and is an indicator of apoptosis, was used. We sought to determine whether pretreating the cells for

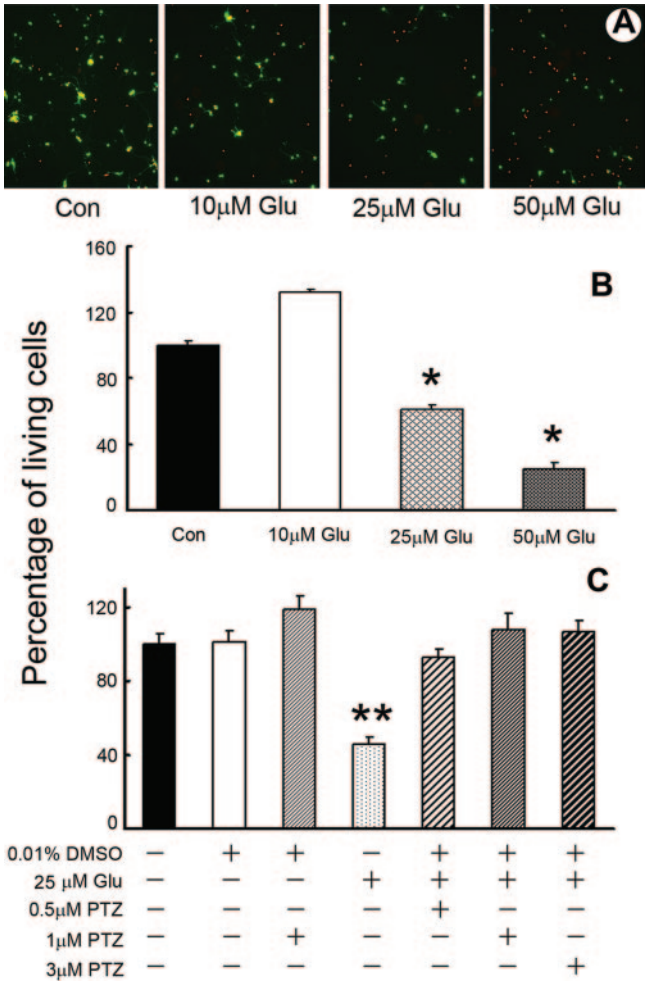

FIGURE 2. Neuroprotective effect of (+)-PTZ on Glu-induced primary GC death. (**A**) Photomicrographs of the viability assay in primary GCs exposed 6 hours to medium with no additional Glu (control) or to medium containing 10 μM, 25 μM, and 50 μM Glu. Living cells fluoresce *green*; dead cells fluoresce *red*. (**B**) Quantitation of live primary GCs after exposure to Glu (10, 25, and 50 μM) for 6 hours. *Significantly different from control and 10 μM Glu; $P < 0.001$. (**C**) Primary GCs were pretreated with (+)-PTZ (0.5, 1, 3 μM) for 1 hour and then were coexposed to 25 μM Glu and (+)-PTZ (0.5, 1, 3 μM) for an additional 16 hours. Cells were subjected to the viability assay to determine viability. **Significantly different from control cells [not treated with Glu] and from cells exposed to Glu and (+)-PTZ; $P < 0.001$. (**B**, **C**) Data are expressed as the mean ± SE of the ratio of living cells to the total number of cells; data were normalized to the control value, which was considered 100% ($n = 10$).

1 hour with varying concentrations of (+)-PTZ followed by a 6-hour cotreatment with 25 μM Glu and (+)-PTZ would protect the cells against death. Figure 3 shows that control cells and cells incubated with 0.01% DMSO (vehicle control) had approximately 10% TUNEL-positive cells. Exposure of cells for 6 hours to 25 μM Glu led to approximately 50% TUNEL-positive cells, consistent with data obtained using the viability assay (Live/Dead assay; Molecular Probes; Fig. 2B). Pretreatment of primary GCs with (+)-PTZ and followed by 6-hour cotreatment with Glu and (+)-PTZ reduced the number of TUNEL-positive cells slightly, but significantly, compared with cells treated with Glu alone. We next sought to determine whether a longer cotreatment time period with Glu and (+)-PTZ would alter the incidence of cell death (Fig. 4). Cells were pretreated with (+)-PTZ (0.5, 1, or 3 μM) for 1 hour, followed by 18-hour coincubation with these concentrations of (+)-PTZ and 25 μM Glu. Figure 4A shows representative photomicrographs of the TUNEL assay when cells received no treatment or were treated with vehicle (0.01% DMSO), Glu (25 μM), or Glu plus (+)-PTZ (0.5, 1, or 3 μM). Green fluorescence reflected cells undergoing apoptosis. Cells treated with increasing dosages of (+)-PTZ showed significantly fewer TUNEL-positive cells. These results were quantified (Fig. 4B), and the data showed that the longer cotreatment time (18 hours) led to a highly significant decrease in the number of TUNEL-positive cells.

To determine whether the protective effects of (+)-PTZ against Glu-induced cell death were associated with alterations in *σR1* gene expression, primary GCs were cultured in the presence of 25 μM Glu for 6 or 18 hours in the presence or absence of 3 μM (+)-PTZ (1-hour pretreatment plus cotreatment). Semiquantitative RT-PCR was performed with RNA isolated from control and treated cells. 18S mRNA was analyzed as an internal control. Data showing RT-PCR amplification of the two bands (465 and 315 bp) representing *σR1* and 18S, respectively, are presented in Figure 5A (6-hour cotreatment) and 5B (18-hour cotreatment). Quantitation of the band densities showed that there were negligible differences in the expression of *σR1* in primary GCs, whether they were exposed to 25 μM Glu for 6 or 18 hours in the presence or absence of (+)-PTZ (data not shown). Although the *σR1* mRNA levels were similar in primary GCs exposed to Glu in the presence or absence of (+)-PTZ, it was not certain that protein levels were comparable. To investigate this, primary GCs were cultured in medium containing 25 μM Glu for 6 or 18 hours in the presence or absence of 3 μM (+)-PTZ (pretreatment 1 hour, plus cotreatment), and immunoblotting was performed. Immunoblotting experiments, using an antibody generated against σR1,[20] detected a band of the appropriate molecular

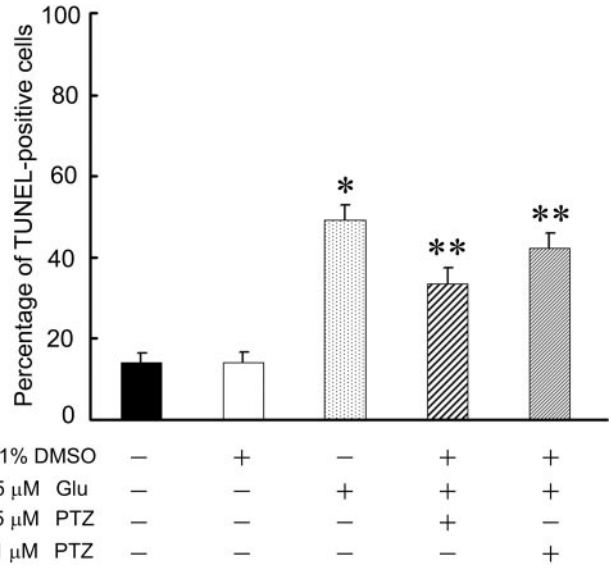

FIGURE 3. Assessment of the number of TUNEL-positive primary GCs after 6-hour incubation with Glu in the absence or presence of (+)-pentazocine ((+)-PTZ). Primary GCs were pretreated for 1 hour with or without (+)-PTZ (0.5 or 1 μM), followed by coincubation for 6 hours with Glu (25 μM) and (+)-PTZ (0.5 or 1 μM); the number of TUNEL-positive cells was determined using the apoptosis detection fluorescein method. Data are expressed as the mean ± SE of the ratio of dead/dying (TUNEL-positive) cells to the total number of cells ($n = 10$). *Significantly different from untreated control or vehicle (DMSO) control at $P < 0.001$. **Significantly different from treatment with 25 μM Glu alone (without (−)-(+)-PTZ).

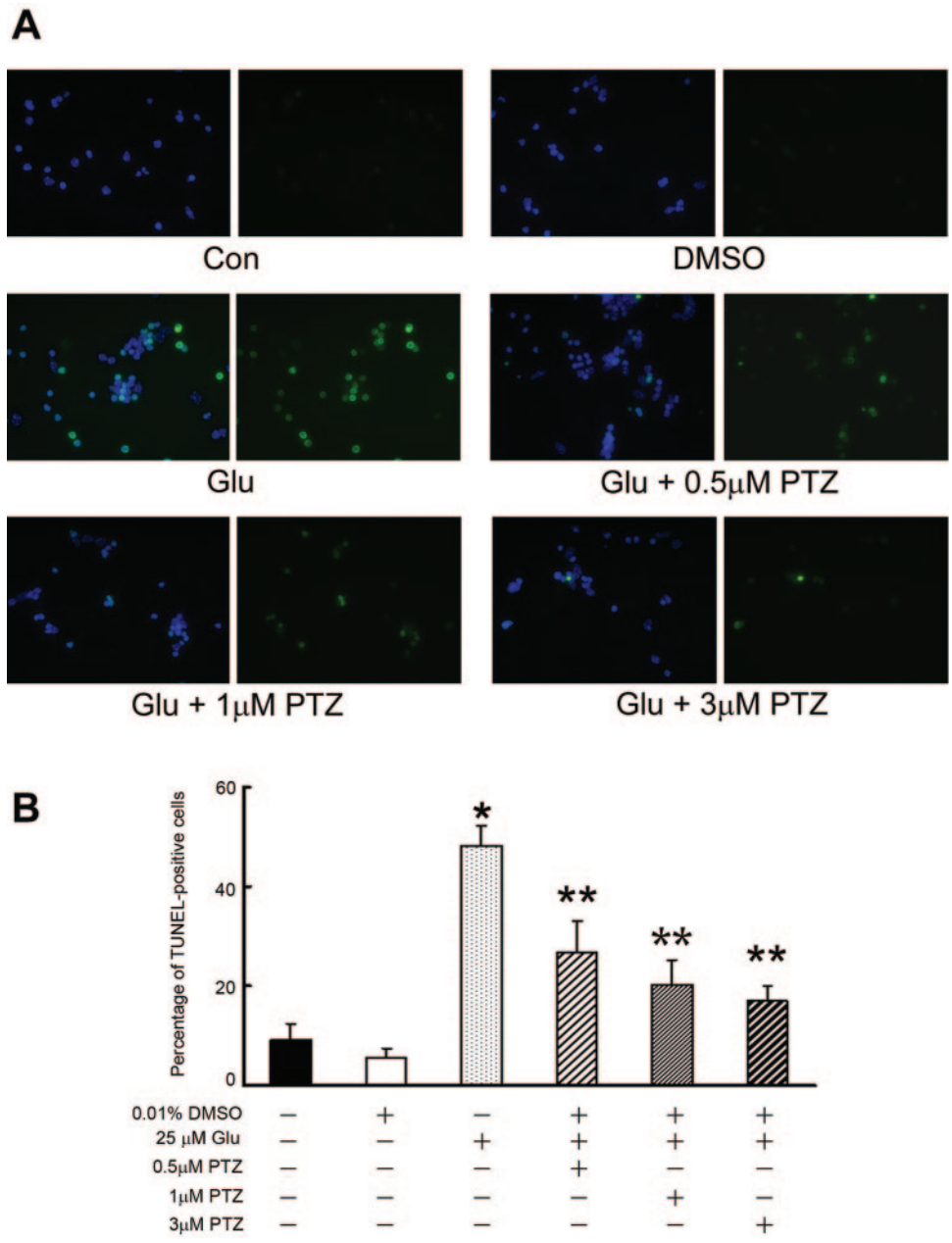

FIGURE 4. Assessment of the number of TUNEL-positive primary GCs when pretreated with (+)-PTZ and then coincubated for 18 hours with Glu and (+)-PTZ. (**A**) Representative photomicrographs showing results of the TUNEL assay. Cells that fluoresce *green* are TUNEL positive, indicative of apoptosis. All samples were labeled also with DAPI (*blue*) to stain nuclei. For each pair of photomicrographs, the *left image* shows the merged (DAPI plus TUNEL staining), and the *right image* shows only the TUNEL staining. *Top*: representative images of cells that received no Glu exposure (control, DMSO vehicle control). *Middle*: marked increase in TUNEL-positive cells after 18-hour exposure to 25 μM glutamate. Remaining three panels show fewer TUNEL-positive cells when pretreated and coincubated with (+)-PTZ (0.5, 1, or 3 μM). (**B**) Quantification of the data from TUNEL analysis shown in (**A**). The number of TUNEL-positive cells was determined per 100 cells counted. Data are expressed as the mean and SE of the ratio of dead/dying cells to the total number of cells ($n = 10$). *Significantly different from control; $P < 0.001$. **Significantly different from treatment with 25 μM Glu alone (without (−)-(+)-PTZ).

weight (M_r approximately 26–27 kDa) in control cells and those treated with 25 μM Glu in the presence or absence of (+)-PTZ and in cells treated with (+)-PTZ alone (Figs. 5C, 5D). Blots were stripped and reprobed with β-actin (M_r approximately 45 kDa) as an internal control. Quantification of the band intensities revealed no significant differences in σR1 protein levels between Glu-treated cells and controls or those treated with (+)-PTZ (data not shown).

In addition to analyzing the effects of Glu on primary GCs, we examined the effects of increasing concentrations of Hcy on primary GC survival and investigated whether (+)-PTZ could alter these effects. Figure 6 shows the dose-response data for primary GCs cultured in the supplemented medium (Neurobasal) for 3 days and then incubated for 6 hours in medium containing increasing concentrations of Hcy (10, 25, 50, and 100 μM). Although earlier studies with the RGC-5 cell line required millimolar concentrations of Hcy to induce cell death,[25] the present experiments using the primary GCs showed that exposure of cells to 10 or 25 μM Hcy led to a slight elevation (approximately 20%) in the number of TUNEL-positive cells compared with untreated controls. When the cells were exposed to 50 μM or 100 μM Hcy, 60% and 80% of the cells were TUNEL positive, respectively. This marked sensitivity of the primary GCs is in keeping with the evidence that micromolar quantities of Hcy can induce apoptotic ganglion cell death in vivo[26] and supports the relevance of the primary GCs in studying Hcy-induced toxicity. Based on these data, subsequent experiments used 50 μM Hcy to induce cell death.

The efficacy of (+)-PTZ as a neuroprotectant in the Hcy-induced primary GC death was tested (Fig. 7). When cells received 1-hour pretreatment with (+)-PTZ, followed by 6-hour cotreatment with 50 μM Hcy and 0.5 or 1 μM (+)-PTZ, there was a slight decrease in the number of TUNEL-positive cells, but the difference did not reach statistical significance (Fig. 7A). However, pretreatment/cotreatment of cells for 6 hours with 3 μM (+)-PTZ led to a significant decrease in the

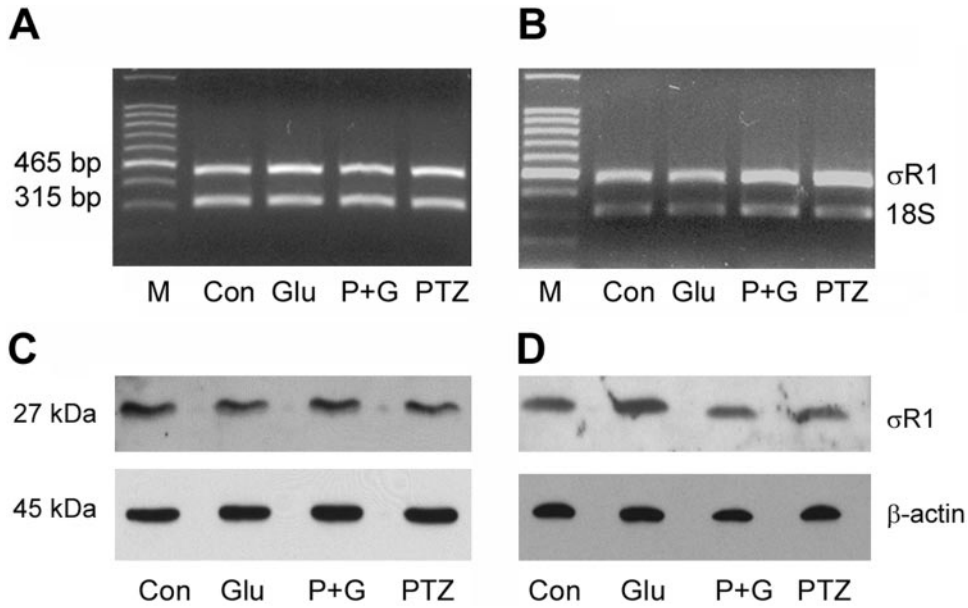

FIGURE 5. σR1 mRNA and protein levels in primary GC after treatment with Glu and (+)-PTZ. Primary GCs were incubated for 6 hours (**A**, **C**) or 18 hours (**B**, **D**) in the absence or presence of 25 μM Glu and in the absence or presence of 3 μM (+)-PTZ. (**A**, **B**) Total RNA was isolated and used for semiquantitative RT-PCR. Primer pairs specific for mouse *σR1* mRNA (465 bp) were used. 18S RNA (315 bp) was analyzed in the same RNA samples as the internal control. RT-PCR products were run on a gel and stained with ethidium bromide. (**C**, **D**) Proteins were extracted from cells and subjected to SDS-PAGE, followed by immunoblotting using an affinity-purified antibody against σR1 (M_r approximately 27 kDa) or β-actin (M_r approximately 45 kDa; internal loading control). M, DNA marker; Con, control; Glu, 25 μM glutamate-treated cells; P + G, (+)-PTZ 3 μM plus 25 μM glutamate; (+)-PTZ, 3 μM (+)-PTZ incubation alone.

number of dying cells (Fig. 7A). When cells were pretreated 1 hour with (+)-PTZ followed by 18-hour cotreatment with 50 μM Hcy in the presence of (+)-PTZ, there was a dose-dependent decrease in the incidence of cell death (Fig. 7B). Primary GCs treated with 1 and 3 μM (+)-PTZ showed a highly significant decrease in the number of TUNEL-positive cells (28% and 25%, respectively, compared with 66% cell death in the Hcy-treated cells). Thus, (+)-PTZ treatment afforded marked protection against neuronal cell death induced by this excitotoxin and Glu.

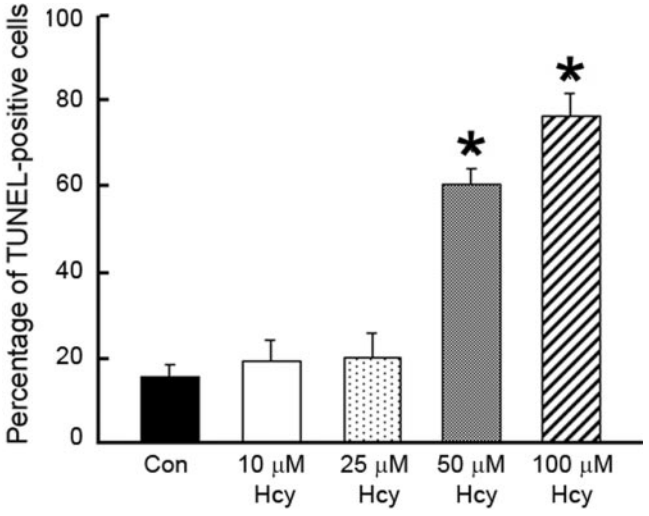

FIGURE 6. Dose-dependent increase in TUNEL-positive primary GCs after exposure to Hcy. The number of TUNEL-positive cells was determined using the apoptosis detection fluorescein method in primary GCs exposed to Hcy (10, 25, 50, and 100 μM) for 6 hours. Data are expressed as the mean ± SE of the ratio of dead/dying cells to the total number of cells ($n = 10$). *Significantly different from control; $P < 0.001$.

As in studies analyzing the effects of excess Glu on σR1 expression (Fig. 5), we examined *σR1* expression after Hcy treatment of cells. RT-PCR showed no difference in the expression of *σR1* mRNA levels, regardless of whether cells were exposed to Hcy for 6 or 18 hours or whether they received (+)-PTZ treatment before Hcy exposure (data not shown). These data were confirmed by real-time RT-PCR. Similarly, there was no difference in σR1 protein levels after the same duration of Hcy exposure in the presence or absence of (+)-PTZ, as examined by Western blot. The data suggest that the neuroprotective effects of (+)-PTZ involved σR1 activation rather than altered σR1 expression.

An advantage of using primary GCs is that the cells develop extensive processes reminiscent of neurons in vivo. This feature can be monitored, and the consequence of exposure to excitotoxins can be evaluated. With the use of DIC microscopy, primary GCs were examined over a period of 18 hours after exposure to 25 μM Glu or 50 μM Hcy (in the absence or presence of (+)-PTZ), and images were captured. A panel of representative photomicrographs is provided in Figure 8. Control cells, which received no excitotoxin treatment, had plump cell bodies typical of healthy neurons that measured approximately 7 to 8 μm in diameter. Intact processes radiated from many cell bodies. Typically, three to five of these processes per cell body extended approximately 10 to 15 μm before they branched into more complex networks. Figure 8 (top row) shows control cells viewed over the 18-hour time period. When cells were treated with Glu, there was progressive loss of the processes. Minimal disruption was evident 3 hours after treatment; however, by 6 hours, the cell bodies were shrunken, the processes were shortened, and the complex

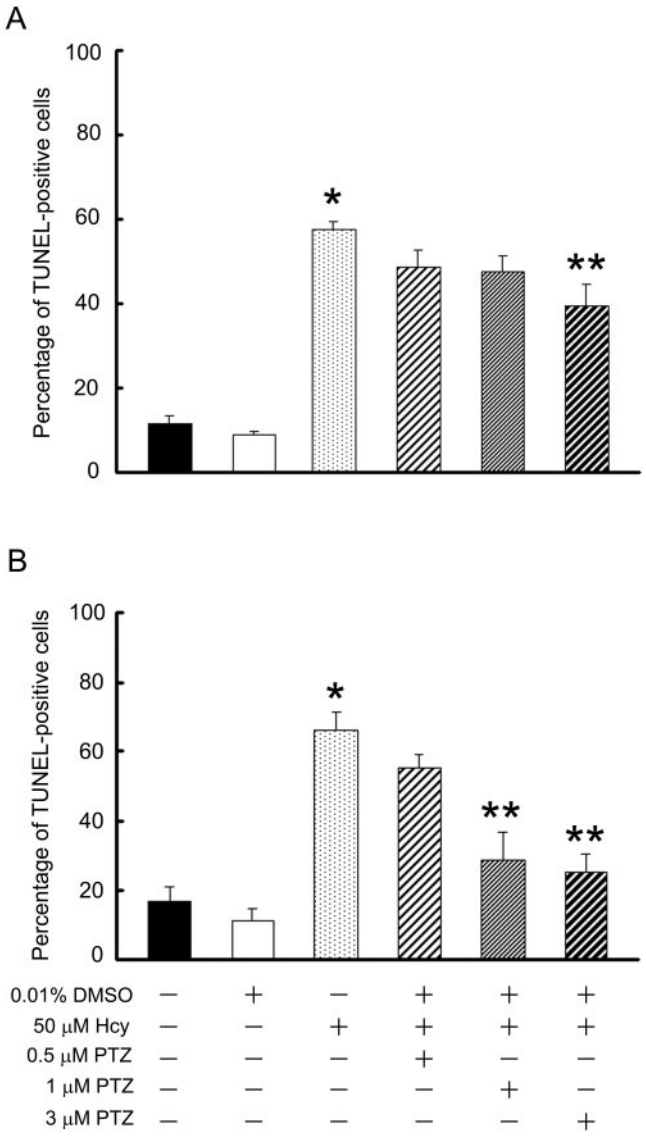

FIGURE 7. Assessment of the number of TUNEL-positive primary GCs after 6- or 18-hour incubation with Hcy and (+)-PTZ. Primary GCs were pretreated for 1 hour with (+)-PTZ (0.5, 1, or 3 μM) followed by coincubation for 6 hours (**A**) or 18 hours (**B**) with Hcy (50 μM) and (+)-PTZ (0.5, 1, or 3 μM). The number of TUNEL-positive cells was determined using the apoptosis detection fluorescein method. Data are expressed as the mean ± SE of the ratio of dead/dying cells to the total number of cells ($n = 10$). *Significantly different from control; $P < 0.001$. **Significantly different from cells treated with 50 μM Hcy; $P < 0.001$.

extensions from the processes were no longer present. By 18 hours, cells treated with Glu were markedly altered in appearance compared with nontreated controls. A few cells retained short processes, but many had no processes at all. Cell bodies were often shriveled and had condensed nuclei. Similarly, when cells were treated with Hcy, disruption of cell fibers was clear, especially after 6-hour treatment; the processes were stubby and appeared clipped. After 18-hour incubation with Hcy, the fibers were either no longer present or had contracted significantly, forming a small network around the cell body. Interestingly, cells pretreated with (+)-PTZ for 1 hour and then cotreated with Glu or Hcy showed marked preservation of neuronal processes. Cell bodies were similar to the nontreated control cells, and the fibers emanated in complex arrays. These morphologic studies supported the TUNEL assays regarding the effects of Glu or Hcy on cell viability. They also demonstrated the profound neuroprotective effects of (+)-PTZ on the cells.

We chose (+)-PTZ because of its potency and its high selectivity as a ligand for σR1.[24,43] Compared with (+)-PTZ, (−)-PTZ has several orders of magnitude less affinity toward σR1. To provide further support that the observed protective effects of (+)-PTZ in primary GCs against excitotoxicity are mediated by σR1, we compared the efficacy of neuroprotection of (+)-PTZ to (−)-PTZ. Primary GCs were pretreated with (+)-PTZ or (−)-PTZ for 1 hour, followed by coincubation with these compounds in the presence of 25 μM Glu. After 18 hours, the cells were subjected to TUNEL assay, and the number of TUNEL-positive cells was determined. As expected, treatment of cells with (+)-PTZ decreased the incidence of TUNEL-positive cells significantly; treatment of cells with (−)-PTZ had no effect on the incidence of apoptosis induced by Glu (Fig. 9). The data demonstrate the selective role of σR1 activation in the observed protective effects.

DISCUSSION

The purpose of this study was to determine whether ligands for σR1 afforded neuroprotection in an excitotoxic model of retinal neuronal death. Earlier studies from our laboratory suggested that the σR1 ligand (+)-PTZ could prevent cell death induced by Glu and Hcy in the RGC-5 (ganglion) cell line. The concentration of the excitotoxins required to induce death in this cell line was high (millimolar range). Although it was considered a first step in assessing neuroprotection by the σR1 ligand, it was not likely to be physiologically relevant because considerably lower concentrations of Glu and Hcy induced ganglion cell apoptosis in vivo.[26] Using methods adapted from rats, we optimized the isolation and maintenance of ganglion cells from neonatal mice[27] and used these cells for the present study. The cells are notable for their expression of neuronal and ganglion cell-specific markers and their extensive processes characteristic of ganglion cells.

Using primary GCs, we first established that σR1 was present in these cells,as it was in the intact mouse retina. Our immunocytochemical and immunoblotting studies showed robust expression of σR1 in these cells. We next established that these cells are exquisitely sensitive to the toxic effects of Glu and Hcy. Approximately 50% of the cells died when exposed to micromolar quantities of Glu (25 μM) or Hcy (50 μM). The active form of Hcy is thought to be the L-isomer; however, Hcy can only be purchased commercially as D,L-homocysteine thiolactone hydrochloride. It is likely that the 50 μM D,L-Hcy used in this study reflected approximately 25 μM of the active (L-isomer form) of the Hcy, underscoring how sensitive the primary ganglion cells are to this excitotoxin. In addition to quantifying the incidence of apoptosis, we were able to visualize the effects of these excitotoxins on cell morphology over the course of the experiment and observed that the cell bodies shrank and the neuronal processes retracted markedly as a result of exposure to these excitotoxins.

The σR1 ligand (+)-PTZ showed marked neuroprotective effects, particularly when the cells were preincubated for 1 hour before exposure to the excitotoxins. Within 6 hours, cell viability improved. Over the 18-hour exposure to (+)-PTZ, the effects were considerable. Interestingly, microscopic examination of the cells revealed marked preservation of the plump cell body and extensive neuronal processes emanating from the cells that had been treated with (+)-PTZ. Pretreatment followed by cotreatment yielded a better result than did cotreatment alone.

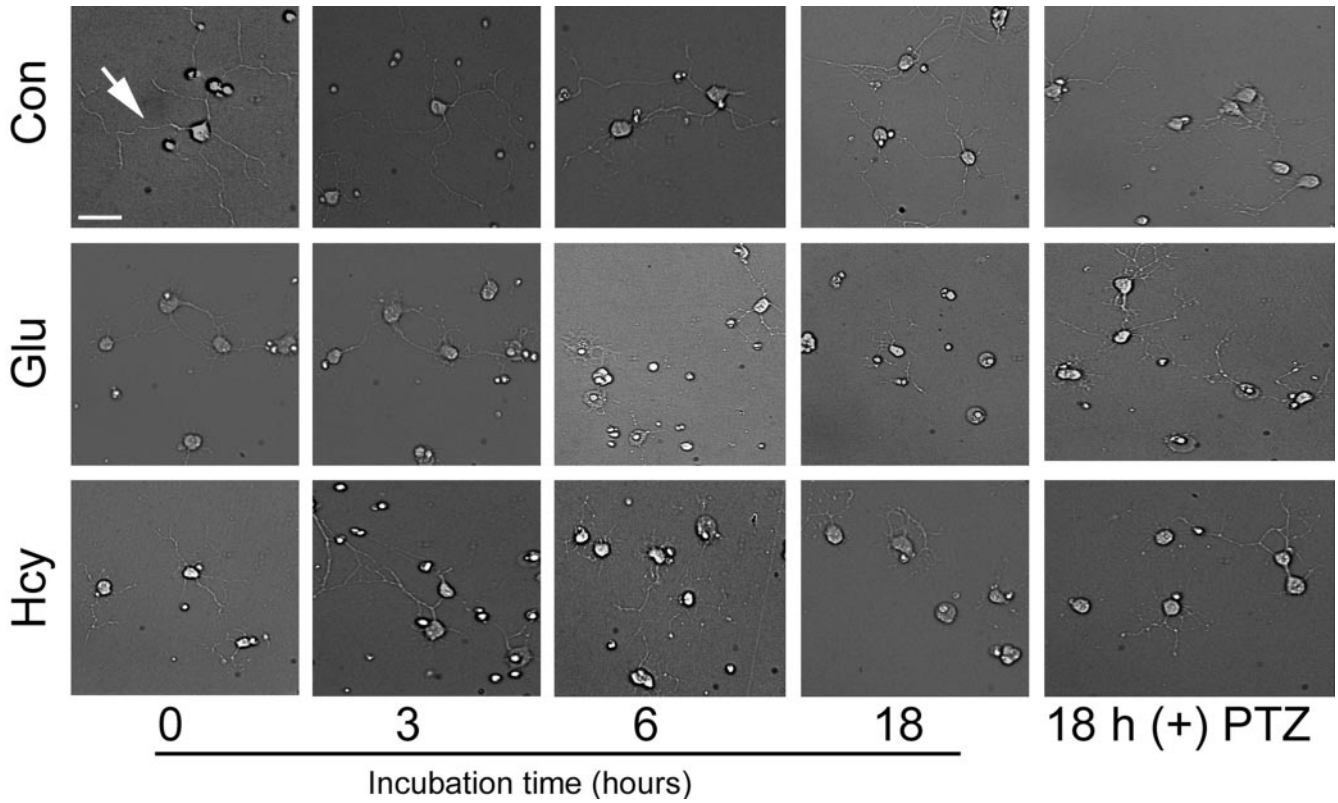

FIGURE 8. Differential interference contrast microscopic analysis of cells exposed to Glu or Hcy and (+)-PTZ. Primary GCs were isolated and cultured as described. Control cells (Con) were not exposed to excitotoxins; the row of images labeled Glu shows cells that were incubated with 25 μM Glu over a period of 18 hours; photomicrographs were acquired at 0, 3, 6, and 18 hours after incubation. The row of images labeled Hcy shows cells that were incubated with 50 μM Hcy over an 18-hour period and photographed at 0, 3, 6, and 18 hours after incubation. In additional experiments, cells were pretreated with (+)-PTZ for 1 hour and then coincubated with (+)-PTZ and the excitotoxin for 18 hours. Cell bodies and processes of cells cotreated with Glu or Hcy and (+)-PTZ were similar in appearance to control cells. *Top left*: control, 0 time. *Arrow* points to a process extending from the cell body. Magnification bar, 15 μm (all photomicrographs are the same magnification).

We also determined whether the neuroprotective effects of (+)-PTZ were mediated by an alteration of σR1 gene or protein expression. RT-PCR analysis and immunoblotting data suggested that they were not, regardless of whether Glu or Hcy was used to induce cell death. It is logical to assume then that the neuroprotective effects were related to activation of σR1 through (+)-PTZ binding. The concentration of (+)-PTZ (500 nM), at which it served as a neuroprotectant in the present study, correlated well with the known affinity of (+)-PTZ for the σR1 (K_d approximately 25 nM).[21,42] Furthermore, the neuroprotective effects of (+)-PTZ were shown to be mediated by σR1 because experiments using (−)-PTZ, the levo-isomer of pentazocine, showed no neuroprotection. Similar findings confirming the specificity of (+)-PTZ for σR1 have been reported in brain.[14]

We showed in earlier studies using primary Müller cells cultured from mouse retina that σR1 binding activity increased when cells were exposed to oxidative stress.[21] Such studies were feasible using the primary Müller cells because, on harvesting, the cells proliferate. This is not the case with the primary GCs. In keeping with their phenotype as a neuronal cell, they do not proliferate. Therefore, it is difficult, if not impossible, to obtain sufficient primary GCs to monitor (+)-PTZ binding unless extraordinary numbers of mice are used. This technical limitation is also a hindrance in studying the signaling events that follow (+)-PTZ-induced σR1 activation. Studies from other laboratories using different model systems showed activation of protein kinase C after (+)-PTZ-induced σR1 activation.[43] Whether this signaling pathway plays a role in (+)-PTZ-mediated protection of primary GCs observed in

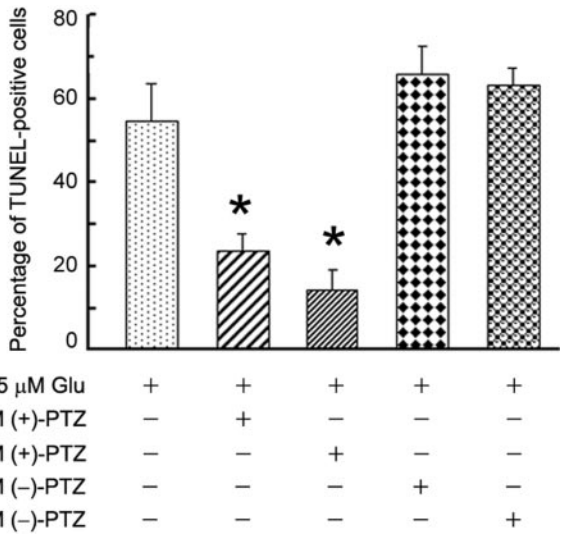

FIGURE 9. Assessment of the number of TUNEL-positive primary GCs pretreated with either (+)-PTZ or (−)-PTZ followed by 18-hour coincubation with Glu. Primary GCs were incubated for 1 hour with (+)-PTZ or (−)-PTZ (0.5, 1 μM) followed by coincubation with PTZ and 25 μM Glu for 18 hours. TUNEL-positive cells were determined using the apoptosis detection kit. The number of TUNEL-positive cells was determined per 100 cells counted. Data are expressed as the mean ± SE of the ratio of dead/dying cells to the total number of cells (n = 10). *Significantly different from treatment with 25 μM Glu alone; $P < 0.001$.

the present study remains to be seen. There is also evidence for protein–protein interaction between σR1 and specific ion channels in the plasma membrane.[44] Binding of ligands to σR1 induces receptor translocation from intracellular sites to the plasma membrane, where protein–protein interaction leads to alterations in the activity of specific ion channels. This would influence intracellular calcium signaling. Thus, potentially diverse mechanisms could mediate the protection of primary GCs from excitotoxicity after (+)-PTZ–induced σR1 activation. We plan to focus in future studies on the signaling events related to σR1 activation in these cells.

The results of this study bring us closer to a physiologically relevant model for exploring the neuroprotective effects of σR1 ligands for retinopathy, particularly ganglion cell death. It is imperative to determine whether the results obtained in this in vitro system are relevant in vivo. Hence, the time is ripe to test σR1 ligands in an animal model in which these cells die, such as is observed in diabetic retinopathy or glaucoma.

Acknowledgments

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